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T-048 P.002/004 F-088

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B. Webb
12/14/01

PATENT
Attorney Docket No.: A-64383-2/RFT/RMS/DHR

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:

CHAN *et al.*

Serial No.: 09/355,214

Filed: July 23, 1999.

For: BLNK PROTEINS

Examiner: S. Zitomer

Group Art Unit: 1655

CERTIFICATE OF MAILING

Transmittal via fax to (703) 746-3148
I hereby certify that this correspondence, including listed enclosures, is being deposited with the United States Postal Service as First Class Mail in an envelope addressed to: Assistant Commissioner of Patents, Washington, DC 20231

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DECLARATION PURSUANT TO 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, DC 20231

Sir:

I, Andrew C. Chan, M.D., Ph.D., do hereby declare as follows:

1. I am a principal investigator at Washington University School of Medicine, Department of Internal Medicine, in St. Louis, MI.

2. I have read and understand the above-identified U.S. patent application Serial No. 09/355,214. I have also read and understand the final Office Action in this case, dated

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8 December 2000. I am familiar with the Examiner's position that the specification does not support that BLNK proteins have a specific and substantial utility. I am also familiar with the Examiner's position that publications of record (attached hereto) concerning BLNK protein function do not support a credible, specific and substantial utility for the claimed BLNK protein compositions.

3. I disagree with the Examiner's position that the specification does not support a specific and substantial utility for the claimed BLNK protein compositions.

4. I disagree with the Examiner's position that publications of record (attached hereto) concerning BLNK protein function do not support a credible, specific and substantial utility for the claimed BLNK protein compositions.

5. The specification asserts a number of characteristics and functions for BLNK proteins which indicate that the claimed BLNK protein compositions have utility. Specifically, given the assertions at page 6, lines 11-15 that BLNK protein is tyrosine phosphorylated by Syk following B cell receptor activation, and at page 20, lines 6-8 that BLNK protein binds to Grb2, PLC γ , Nck and Vav, and regulates calcium levels and modulates cytoskeletal organization, and at page 19, lines 28-29 that BLNK protein is critical for B cell receptor mediated response and B cell function, I would expect to be able to use the claimed BLNK protein compositions for a specific, credible, real-world utility, for example as outlined in the specification at page 23, lines 4-11 which describes the use of BLNK protein for screening bioactive agents for an ability to modulate BLNK protein activity.

6. Attached hereto are two published journal articles of record concerning BLNK protein function, Pappu et al., Science 286:1949-1954, 1999, and Minegishi et al., Science 286:1954-1957, 1999. I am senior author on the former paper and a co-author on the latter paper. The results disclosed in these two papers confirm the accuracy of my expectations from reading

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the present application. Specifically, Pappu et. al disclose BLNK gene function is required for normal B cell development, while Minegishi et. al. disclose that loss of BLNK gene function in humans is associated with early onset hypogammaglobulinemia and the absence of mature B cells. These results support the assertion set forth in the instant specification that BLNK proteins are critical for B cell function, and further support that the claimed BLNK protein compositions have credible, specific and substantial utility.

7. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that the making of willful false statements and the like are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful statements may jeopardized the validity of the application or any patent issuing thereon.

Date: November 1, 2001

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Table 2. Effects of simvastatin on trabecular bone volume and bone formation rates. Simvastatin was given in doses of 5 to 50 mg/kg/day by oral gavage for 35 days to (i) 3-month-old virgin female rats (experiment 1), (ii) 3-month-old virgin female rats that had been ovariectomized within 7 days after the start of treatment (experiment 2), and (iii) 3-month-old virgin female rats that had been ovariectomized 2 months before treatment (experiment 3). In each experiment, the rats were weight matched and divided into treatment groups of 10. The rats were lightly anesthetized with isoflurane before ovariectomy. Animals were pair fed throughout the experimental period and body weights were determined weekly. Values in parentheses are percent change from vehicle-treated controls. BV/TV, bone volume/tissue volume; Ocl, osteoclasts; BFR, bone formation rate; OVX/veh, ovariectomized rats treated with vehicle; hPTH, human PTH; ND, not determined.

Treatment	Trabecular bone volume (% BV/TV)	BFR ($\mu\text{m}^3/\mu\text{m}^2/\text{day}$)	No. of Ocl/mm ² of bone surface
Experiment 1			
Control	13.4 \pm 1.4		13.7 \pm 1.2
Simvastatin (10 mg/kg/day)	18.6 \pm 1.4* (+39)	ND	11.6 \pm 1.4 (-15)
hFGF-1 (100 $\mu\text{g}/\text{kg}/\text{day}$)	21.4 \pm 1.7* (+60)	ND	7.5 \pm 1.3* (-45)
Experiment 2			
OVX/veh	6.9 \pm .87	0.6 \pm 0.1	8 \pm 0.2
Simvastatin (1 mg/kg/day)	8.6 \pm .41 (+25)	ND	ND
Simvastatin (10 mg/kg/day)	13.4 \pm 2* (+94)	1.2 \pm .11 (100*)	7 \pm 0.3 (-12.5)
Experiment 3			
OVX/veh	4.6 \pm 0.58	0.151 \pm 0.01	1.2 \pm 0.1
Simvastatin (5 mg/kg/day)	9 \pm 0.8* (+96)	0.196 \pm .021* (30)	0.9 \pm 0.1 (-25)
Simvastatin (10 mg/kg/day)	8.6 \pm 0.9* (+87)	0.229 \pm .034* (52)	0.78 \pm .06* (-33)
hPTH (80 $\mu\text{g}/\text{kg}/\text{day}$)	20 \pm 1.9* (+348)	0.228 \pm .025* (51)	0.84 \pm 0.15 (-30)

*Significantly greater than control ($P < 0.01$).

with this process may lead to osteoclast apoptosis and cessation of bone resorption (18, 20). We cannot exclude the possibility that the statins both inhibit bone resorption and promote bone growth, and we did observe a concomitant decrease in osteoclast numbers (Table 2). However, this effect appeared minor in comparison to the effect on new bone formation and osteoblast maturation.

The statins used in our studies and currently on the market are not ideal for use as systemic bone-activation agents. These statins were selected for their capacity to lower serum cholesterol, which requires targeting to HMG Co-A reductase in hepatic cells. Thus, the concentration of statin in other tissues is much lower than in the liver. The most efficacious statins would be those that distribute themselves to the bone or bone marrow. A preliminary retrospective analysis of older women taking lipid-lowering agents suggests that statin use is accompanied by greater hip bone mineral density and lower risk of hip fractures (relative risk = 0.30) (21); however, the sample size (598 statin users) was too small to yield definitive information.

The most powerful anabolic agents for bone are the peptide growth factors intrinsic to the tissue. For example, systemically administered FGF-1 restores trabecular microarchitecture and increases bone volume (15). However, all of the peptide growth factors have disadvantages—they can be mitogenic to other bone cells and nonselective in their effects. In addition, the FGFs cause hypotension, which limits their potential use in elderly patients (22).

Our results suggest that statins, which are

orally bioavailable and have been safely administered to patients for more than a decade, may merit further investigation as potential anabolic agents for bone. When the doses are extrapolated from humans to rats with respect to lipid lowering, the statins' effects on bone occur at doses similar to the lipid-lowering doses used in humans.

References and Notes

1. L. J. Melton III, *J. Bone Miner. Res.* **10**, 175 (1995).
2. S. E. Harris et al., *Mol. Cell. Differ.* **3**, 137 (1995); D. Chen et al., *Calcif. Tissue Int.* **60**, 283 (1997).
3. S. E. Harris et al., *J. Bone Miner. Res.* **9**, 855 (1994).
4. N. Ghosh-Choudhury et al., *Endocrinology* **137**, 331 (1996).
5. J. E. Rossouw, B. Lewis, B. M. Rifkind, *N. Engl. J. Med.* **323**, 1112 (1990).
6. M. R. Law, N. J. Wald, S. G. Thompson, *Br. Med. J.* **308**, 367 (1994).
7. I. R. Garrett and G. R. Mundy, data not shown.
8. S. Rao et al., *Proc. Natl. Acad. Sci. U.S.A.* **96**, 7797 (1999).
9. M. Gowen and G. R. Mundy, *J. Immunol.* **136**, 2478 (1986).
10. K. Traianedes, M. R. Dallas, I. R. Garrett, G. R. Mundy, L. F. Bonewald, *Endocrinology* **139**, 3178 (1998).
11. B. F. Boyce, T. B. Aufdemorte, I. R. Garrett, A. J. P. Yates, G. R. Mundy, *Endocrinology* **125**, 1142 (1989).
12. M. Sabatini, B. Boyce, T. Aufdemorte, L. Bonewald, G. R. Mundy, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 5235 (1988).
13. A. J. P. Yates et al., *J. Clin. Invest.* **81**, 932 (1988).
14. C. Marcelli, A. J. P. Yates, G. R. Mundy, *J. Bone Miner. Res.* **5**, 1087 (1990).
15. C. R. Dunstan et al., *J. Bone Miner. Res.*, in press.
16. R. L. Jilka et al., *Science* **257**, 88 (1992).
17. S. P. Luckman et al., *J. Bone Miner. Res.* **13**, 581 (1998).
18. M. J. Rogers et al., *Mol. Pharmacol.* **47**, 398 (1995).
19. J. E. Fisher et al., *Proc. Natl. Acad. Sci. U.S.A.* **96**, 133 (1999).
20. M. J. Rogers et al., *J. Bone Miner. Res.* **11**, 1482 (1996).
21. D. C. Bauer et al., *J. Bone Miner. Res.* **14** (suppl. 1), 1188 (1999).
22. P. Cuevas et al., *Science* **254**, 1208 (1991).
23. We thank L. Knieriem, L. A. Trafton, and N. Garrett for the preparation of this manuscript.

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Requirement for B Cell Linker Protein (BLNK) in B Cell Development

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Linker proteins function as molecular scaffolds to localize enzymes with substrates. In B cells, B cell linker protein (BLNK) links the B cell receptor (BCR)-activated Syk kinase to the phosphoinositide and mitogen-activated kinase pathways. To examine the *in vivo* role of BLNK, mice deficient in BLNK were generated. B cell development in BLNK^{-/-} mice was blocked at the transition from B220⁺ CD43⁺ progenitor B to B220⁺ CD43⁻ precursor B cells. Only a small percentage of immunoglobulin M⁺ (IgM⁺), but not mature IgM⁺ IgD⁺ B cells were detected in the periphery. Hence, BLNK is an essential component of BCR signaling pathways and is required to promote B cell development.

Engagement of the BCR activates distinct families of cytoplasmic protein tyrosine kinases (PTKs) to phosphorylate enzymes that

are required for the generation of second messengers (1). In turn, the coordinate generation of second messengers is important for normal B cell function because disruption of selected signaling pathways is associated with B cell anergy (2). Linker or adapter molecules play integral roles in linking the BCR-activated PTKs with these enzymes. One such linker molecule, BLNK (also known as SLP-65, BASH, and BCA), is phos-

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phorylated by Syk after BCR activation and interacts with enzymes, including phospholipase C- γ , Bruton's tyrosine kinase, and Vav (a guanine nucleotide exchanger for the Rho-GTPases), as well as the Grb2 and Nck linker proteins (3–5). An essential role for BLNK in BCR activation was demonstrated in a chicken *BLNK*^{-/-} DT40 cell line that cannot increase the intracellular calcium concentration ([Ca²⁺]_i) or efficiently activate the Erk-, JNK-, and p38-mediated signaling pathways (6).

To better define the expression pattern of BLNK, we developed an intracellular fluorescence-activated cell sorting (FACS) staining assay for BLNK. Consistent with earlier reports (3, 5), BLNK expression was detected in peripheral B, but not T, lymphocytes (7) (Fig. 1A). Analysis of bone marrow-derived cells showed the highest BLNK expression in early development, with progressively lower

expression during B cell maturation (7, 8) (Fig. 1B). Hence, BLNK is expressed throughout B cell ontogeny and suggests a potential role for BLNK in B cell development, maturation, or function.

To investigate the *in vivo* role of BLNK, we undertook a gene-targeting approach to generate and analyze *BLNK*^{-/-} mutant mice. Because BLNK is a substrate of Syk and *syk*^{-/-} mice hemorrhage extensively in utero and die during the perinatal period (3, 9), we were concerned that *BLNK*^{-/-} mice might suffer a similar fate. In addition, gene targeting of the BLNK homolog, *SLP-76*, results in mice that die from hemorrhage caused by a defect in collagen-induced platelet aggregation (10). To circumvent the embryonic lethality that may be encountered in germ line knockout mice, we also used the *RAG2*^{-/-} blastocyst complementation system to assay for BLNK function in lymphocytes (11). The

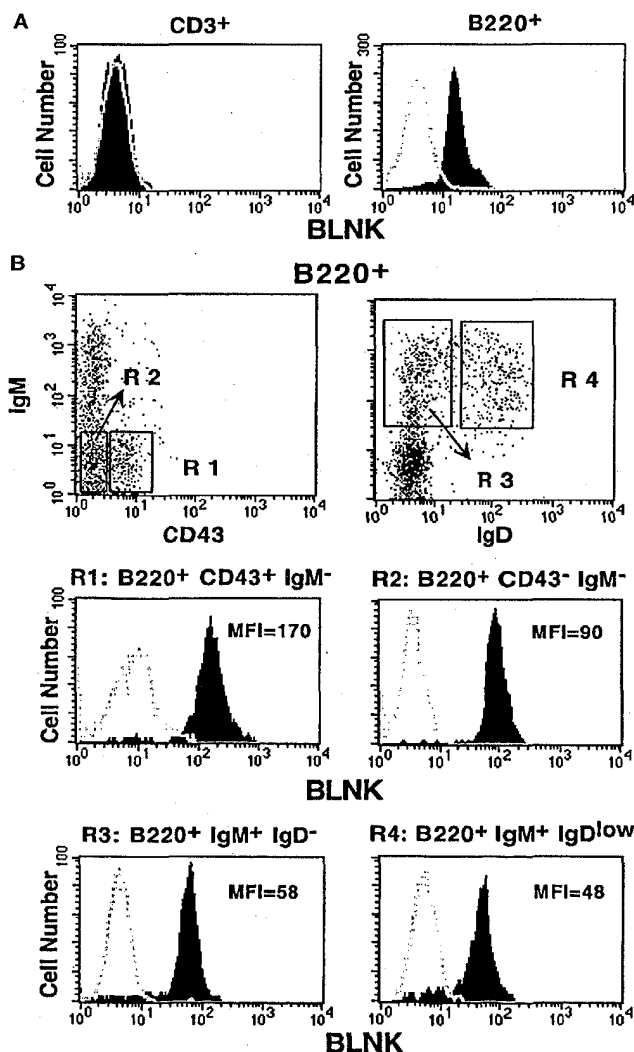
data presented here represent analyses from both approaches.

Disruption of *BLNK* was accomplished by a targeted mutation of exon 1, which encodes amino acids 1 through 60, including the initiation codon (12) (Fig. 2A). For *RAG2*^{-/-} blastocyst complementation, the *BLNK*⁺ allele in the *BLNK*^{+/+} embryonic stem (ES) clone was further targeted with a puromycin selection cassette to generate *BLNK*^{-/-} ES cells (13) (Fig. 2B). To generate germ line mutant mice, *BLNK*^{-/-} ES cells were injected into B6 blastocysts to yield chimeras that were then crossed with wild-type B6 mice to generate *BLNK*^{+/+} germ line mutants. The genotypes of the mature offspring from such crosses occurred at the expected Mendelian ratios and the *BLNK*^{-/-} mutation did not incur any embryonic or perinatal lethality (14). Furthermore, mature *BLNK*^{-/-} mice were healthy under specific pathogen-free conditions and did not display any evidence of gross hemorrhaging (14). To evaluate the developmental potential of *BLNK*^{-/-} ES cells, we also injected these cells into *RAG2*^{-/-} blastocysts to generate chimeras (*R2:BLNK*^{-/-}) for direct analysis (15).

To assess the effect of the mutation on BLNK expression, we used the Ly 9.1 surface marker to distinguish between bone marrow cells derived from the *RAG2*^{-/-} blastocyst (Ly 9.1⁻) and the *BLNK*^{-/-} ES cells (Ly 9.1⁺) (16). Whereas the B220⁺ Ly 9.1⁺ cells from wild-type 129 mice and B220⁺ Ly 9.1⁺ cells from *RAG2*^{-/-} mice expressed BLNK (Fig. 2D, left two bottom panels), no BLNK was detected in the B220⁺ Ly 9.1⁺ cells isolated from the *R2:BLNK*^{-/-} chimeras, as assessed by intracellular staining (Fig. 2D, bottom right panel). Similar to the results from the *R2:BLNK*^{-/-} chimerae, no BLNK protein was detected in cell lysates of total bone marrow from germline *BLNK*^{-/-} mice (17) (Fig. 2E).

The effects of BLNK deficiency on lymphocyte development *in vivo* was examined by analyzing cells isolated from primary and secondary lymphoid organs. Consistent with the absence of BLNK expression in T cells, T cell number, development, and function were normal in both *BLNK*^{-/-} germ line and *R2:BLNK*^{-/-} chimeric mice (14, 18–20). In contrast, an ~65% reduction in splenocyte number was found in *BLNK*^{-/-} mice as compared to *BLNK*^{+/+} or *+/+* mice (18). Because the development and function of peripheral T cells were normal (14, 19), we further investigated the nature of this defect by analyzing the B cell compartment in primary and secondary lymphoid organs. Although the numbers of cells recovered from the bone marrow of *BLNK*^{+/+} and *BLNK*^{-/-} mice were similar (18), bone marrow cells from *BLNK*^{-/-} germ line and *R2:BLNK*^{-/-} chimeric mice displayed a profound block in B cell devel-

Fig. 1. Expression of BLNK in lymphocyte development. (A) BLNK is expressed in murine B, but not T, cells. CD3⁺ (left panel) or B220⁺ (right panel) splenocytes isolated from C57BL/6 mice were analyzed by intracellular staining with an antiserum to BLNK (shadowed areas) or preimmune serum (solid line) (7). (B) BLNK expression during murine B cell development. Bone marrow-derived cells isolated from C57BL/6 mice were analyzed with four-color FACS analysis (8). Cells stained for B220, IgM, and CD43 (left panel) or for B220, IgM, and IgD (right panel) were analyzed as described above. Each developmental subset—B220⁺ CD43⁺ IgM⁻ (pro-B cells; R1), B220⁺ CD43⁻ IgM⁻ (pre-B cells; R2), B220⁺ IgM⁺ IgD⁻ (immature B cells; R3), and B220⁺ IgM⁺ IgD^{low} (mature B cells; R4)—was analyzed for BLNK expression (7).



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opment. *BLNK*^{-/-} mice accumulated B220⁺CD43⁺ progenitor B cells (pro-B cells) (21) (Fig. 3A). Consistent with the presence of pro-B cells, the levels of V_H to DJ_H recombination were comparable in *BLNK*^{+/-} and *BLNK*^{-/-} bone marrow-derived cells (14). *BLNK*^{-/-} mice had CD43⁺ pro-B cells but failed to develop B220^{hi}CD43⁻ B cells, although a small percentage of B220⁺CD43⁻ B cells was present (10.0 ± 8.7% for *BLNK*^{-/-} versus 44.4 ± 14% for *BLNK*^{+/-} or *+/+*, *P* < 0.001, *n* = 11) (20) (Fig. 3A). Because the transition from the CD43⁺ to CD43⁻ stage is normally associated with a decrease in cell size, as measured by the forward scatter value (8), the B220⁺CD43⁻ B cells isolated from *BLNK*^{-/-} mice remained large, in contrast to the smaller B220⁺CD43⁻ B cells from *BLNK*^{+/-} mice (14). In addition, the *BLNK*^{-/-} bone marrow (B lineage) cells failed to progress efficiently from the immature B220^{lo}IgM^{lo} (immunoglobulin M, IgM) stage to transitional B220^{lo}IgM^{hi} or mature B220^{hi}IgM⁺ stages (1.3% ± 0.9% in *BLNK*^{-/-} mice for the latter two stages versus 13.1 ± 5% in *BLNK*^{+/-} or *+/+* mice, *P* < 0.001, *n* = 10) (20) (Fig. 3A). The small percentage of IgM^{lo} bone marrow B cells

that develop in *BLNK*^{-/-} mice express a mature surface BCR because many are also Igκ⁺ (14).

Analysis of splenocytes revealed a substantial decrease in the numbers of IgM⁺ peripheral B cells (2.4 ± 2.6% for *BLNK*^{-/-} versus 30.7 ± 6.2% for *BLNK*^{+/-} or *+/+*, *P* < 0.001, *n* = 10) (20) (Fig. 3B). As in the bone marrow, the few *BLNK*^{-/-} IgM⁺ B cells found in the spleen were also larger in size than IgM^{hi} *BLNK*^{+/-} B cells (14). Concomitant with the profound decrease in peripheral B cells in the spleen, IgM⁺ B cells were also reduced in the lymph node (Fig. 3C). Hence, the absence of BLNK results in a developmental block that leads to reduced numbers of IgM⁺ cells in the periphery. Older *BLNK*^{-/-} mice (8 to 13 weeks old) showed increased numbers of B220⁺ IgM⁺ B cells [(1.4 ± 1.2) × 10⁶ B220⁺ IgM⁺ cells, *n* = 8] as compared to younger *BLNK*^{-/-} mice [3 to 6 weeks old; (0.58 ± 0.31) × 10⁶, *P* < 0.001, *n* = 9]. In spite of this accumulation, these older *BLNK*^{-/-} mice still have more than 10 times fewer B220⁺ IgM⁺ B cells than their age-matched *BLNK*^{+/-} or *+/+* counterparts [(19 ± 6.7) × 10⁶ B220⁺ IgM⁺

cells in older *BLNK*^{+/-} or *+/+* mice, *n* = 6, versus (1.4 ± 1.2) × 10⁶ B220⁺ IgM⁺ cells in older *BLNK*^{-/-} mice, *P* < 0.001, *n* = 8] (20).

Analysis for mature B cells revealed a marked reduction of B220^{hi}IgM⁺ cells (<1%) in the bone marrow of young and old *BLNK*^{-/-} mice (Fig. 3A) (14). Mature IgM^{lo}IgD^{hi} cells were similarly reduced (<1%) in the periphery of young and old *BLNK*^{-/-} mice (Fig. 4, A and B). Staining with CD21 revealed the presence of CD21⁺IgM^{hi} T2 transitional B cells and a reduction of CD21⁺IgM^{lo} mature B cells (<1%) in *BLNK*^{-/-} mice (22) (Fig. 4B). Consistent with the decrease in mature B cells, serum Ig in older *BLNK*^{-/-} mice was significantly reduced as compared to the amount in wild-type mice (23) (Fig. 4C).

The *BLNK*^{-/-} B cells that accumulated in the periphery of older mice further revealed a maturation defect in these cells. In contrast to *BLNK*^{+/-} mice, in which transitional B220⁺IgM⁺IgD⁺ B cells develop into mature B220^{hi}IgM^{lo}IgD^{hi} B cells, and in contrast to the B cells that accumulate in the λ5^{-/-} mice (24), *BLNK*^{-/-} splenic B cells are larger in size and express higher mem-

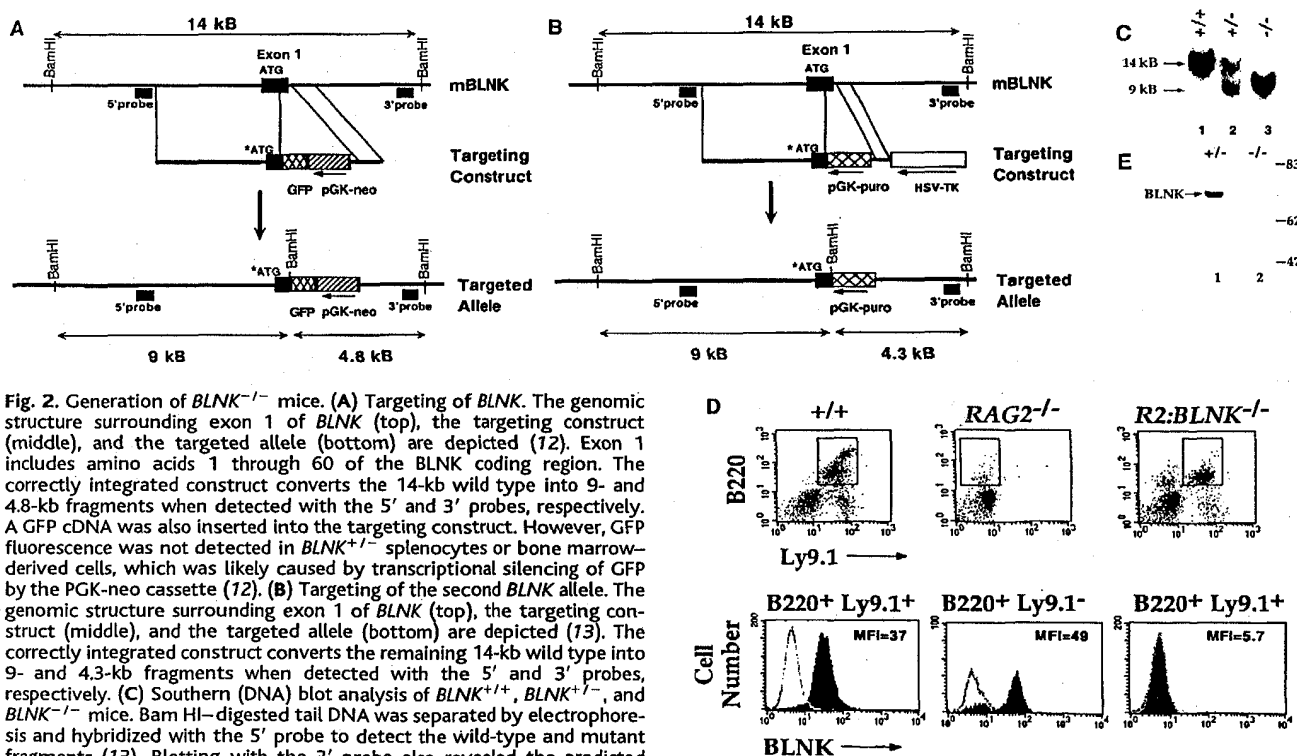


Fig. 2. Generation of *BLNK*^{-/-} mice. (A) Targeting of *BLNK*. The genomic structure surrounding exon 1 of *BLNK* (top), the targeting construct (middle), and the targeted allele (bottom) are depicted (72). Exon 1 includes amino acids 1 through 60 of the BLNK coding region. The correctly integrated construct converts the 14-kb wild type into 9- and 4.8-kb fragments when detected with the 5' and 3' probes, respectively. A GFP cDNA was also inserted into the targeting construct. However, GFP fluorescence was not detected in *BLNK*^{+/-} splenocytes or bone marrow-derived cells, which was likely caused by transcriptional silencing of GFP by the pGK-neo cassette (72). (B) Targeting of the second *BLNK* allele. The genomic structure surrounding exon 1 of *BLNK* (top), the targeting construct (middle), and the targeted allele (bottom) are depicted (13). The correctly integrated construct converts the remaining 14-kb wild type into 9- and 4.3-kb fragments when detected with the 5' and 3' probes, respectively. (C) Southern (DNA) blot analysis of *BLNK*^{+/-}, *BLNK*^{-/-}, and *BLNK*^{-/-} mice. Bam HI-digested tail DNA was separated by electrophoresis and hybridized with the 5' probe to detect the wild-type and mutant fragments (13). Blotting with the 3' probe also revealed the predicted mutant 4.8-kb fragment in *BLNK*^{+/-} and *BLNK*^{-/-} mice (14). (D) Absence of BLNK protein in *RAG2*^{-/-} chimeric mice. B220⁺ bone marrow cells from 129 wild-type (Ly9.1⁺; left panels), *RAG2*^{-/-} (Ly9.1⁻; middle panels), or *R2:BLNK*^{-/-} chimeric (Ly9.1⁺; right panels) mice were analyzed by intracellular staining for BLNK as described in Fig. 1A (7). (E) Absence of BLNK protein in *BLNK*^{-/-} bone marrow-derived cells. Bone marrow-derived cells from germ line *BLNK*^{+/-} (lane 1) and *BLNK*^{-/-} (lane 2) littermates were immunoblotted with an antiserum to BLNK (17). Equal loading of cell lysates was confirmed by immunoblotting with an antiserum to actin (Sigma) (14).

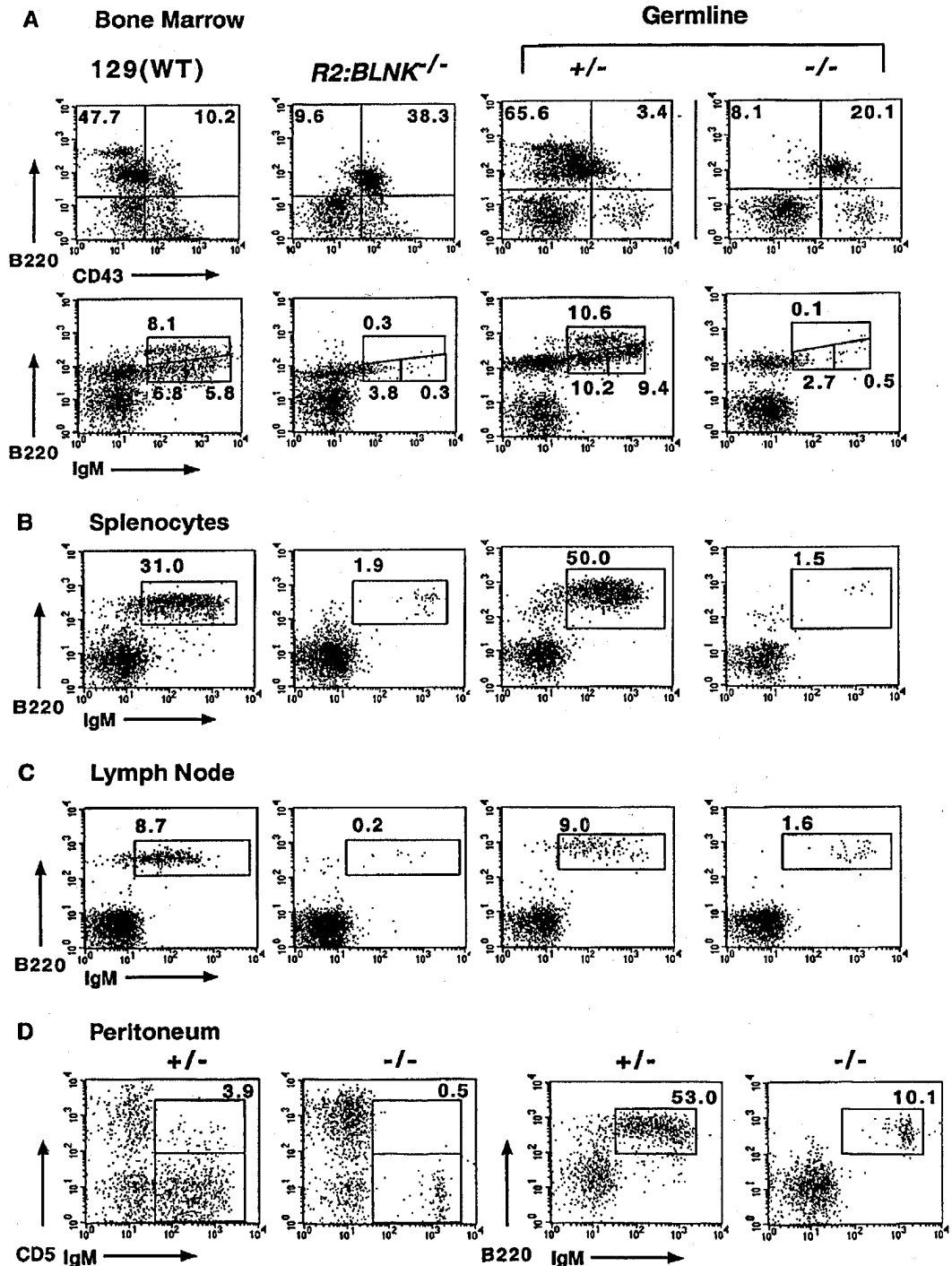
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brane IgM (Fig. 4B) (14). These IgM^{hi} cells may represent B cells that have matured through the pro- to precursor B cell (pre-B cell) transition but are blocked in IgM signaling and, therefore, accumulate as large IgM^{hi} cells. Alternatively, these cells may result from a selection bias in which B cells can

bypass the absence of BLNK by increasing membrane Ig expression and decreasing the signaling threshold. As CD45^{-/-} immature B cells expressing a transgenic BCR can be rescued from death by chronic exposure to antigen (25), heightened BCR signaling may bypass the requirement for CD45. Similarly,

BLNK deficiency may abolish development and result in the death of most B cells, except those that express very large amounts of IgM, which partially compensates for the signaling defect incurred by BLNK deficiency. The IgM⁺⁺ BLNK^{-/-} B cells could increase free cytoplasmic calcium after BCR cross-linking,

Fig. 3. B cell development in BLNK^{-/-} mice. Cells isolated from bone marrow (A), spleen (B), and lymph nodes (C) of 3- to 5-week-old animals were stained with the antibodies indicated in each figure and analyzed by FACS analysis (21). Data from both R2:BLNK^{-/-} chimeric [left two panels for (A) through (C)] and germ line [right two panels for (A) through (C)] mice are shown. In the RAG2^{-/-} blastocyst complementation assay, 129 wild-type and RAG2^{-/-} age-matched mice were analyzed in parallel as controls (14). No differences were detected between BLNK^{+/+} and BLNK^{+/-} mice (14). The percentages of gated cells are indicated. These analyses were representative of a minimum of five pairs each of RAG2^{-/-} chimeric and germ line animals. Experiments from both approaches produced similar results. (D) Peritoneal cells isolated from 6- to 13-week-old mice were stained with the antibodies indicated and analyzed by FACS analysis (21). Cell recoveries were comparable in yield from BLNK^{+/+} and BLNK^{-/-} mice [4.5×10^6 for BLNK^{+/+} and $(3.2 \pm 0.6) \times 10^6$ for BLNK^{-/-} mice, $n = 5$].



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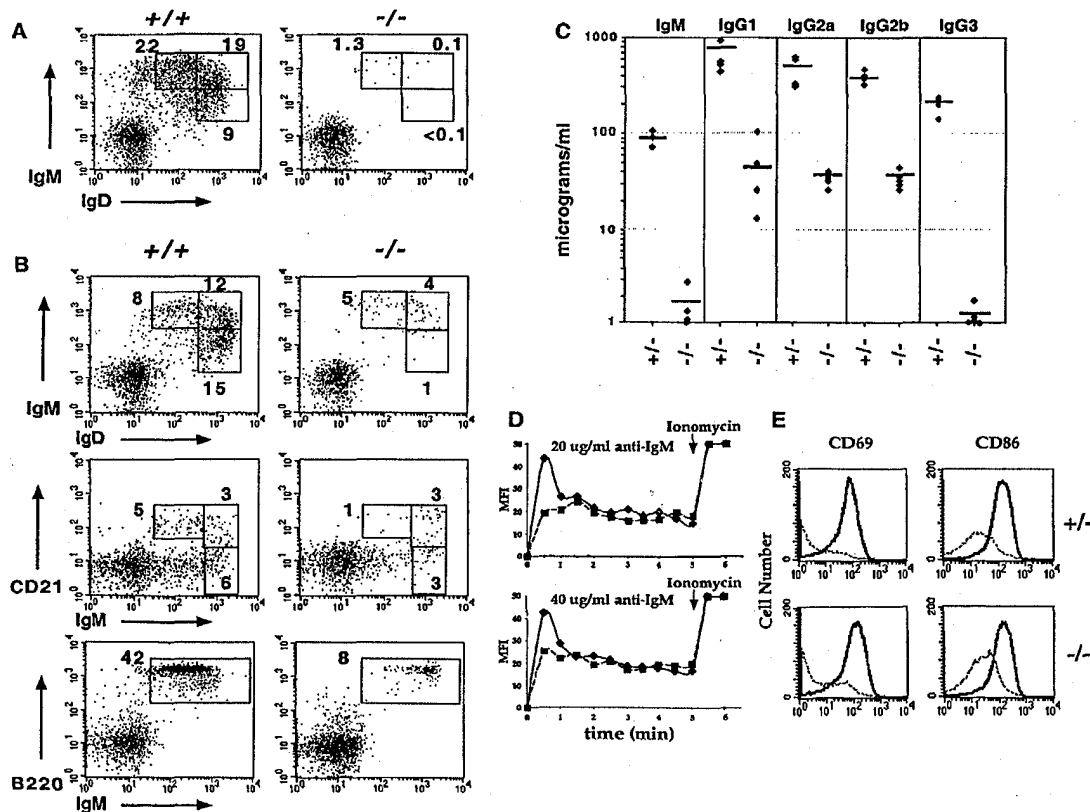
albeit less efficiently than wild-type cells, despite expressing 10- to 50-fold more membrane IgM than *BLNK*^{+/+} B cells (26) (Fig. 4D). In addition, *BLNK*^{-/-} B cells also up-regulate CD69 and CD86 cell surface activation markers after BCR cross-linking (27) (Fig. 4E). Hence, these large IgM⁺ *BLNK*^{-/-} B cells are capable of some BCR-mediated signaling functions.

We also analyzed the development of a distinct subset of B cells known as B-1a cells. These cells are distinguished from conventional B-2 cells by their expression of CD5 and their capacity for self-renewal (28). Whereas *BLNK*^{+/+} and *BLNK*^{-/-} mice had comparable numbers of peritoneal cells, *BLNK*^{-/-} mice had a substantial decrease in the CD5⁺ IgM⁺ B-1a B cell population (<1%) in the peritoneum in young and old mice (Fig. 3D) (14). In addition, CD5⁺ CD11b⁺ IgM⁺ B-1b B cells were also absent (<0.5%) in the peritoneum and the spleen of *BLNK*^{-/-} mice (14, 29). Hence, BLNK is also required for development of B-1 cells.

Because signals from both the pre-B and IgM BCRs are required for normal B cell development (1, 30), these studies showed the critical role of BLNK in the development of IgM⁺ cells. Similar to *syk*^{-/-} mice (9), the absence of BLNK also compromises pre-BCR function to affect the development of B220⁺ CD43⁻ B cells that, in turn, limit their differentiation into B220^{hi} IgM⁺ B cells in the bone marrow. As a result, few B cells are present in the periphery. However, whereas the IgM⁺ B cells that develop in *syk*^{-/-} mice express little membrane IgM (9), the B cells that accumulate in *BLNK*^{-/-} mice express large amounts of membrane IgM (Fig. 4B). This difference suggests that additional substrates of Syk might exist to partially transduce pre-BCR signals in the absence of BLNK. In accordance with this, the IgM⁺ B cells that accumulate in the periphery of older *BLNK*^{-/-} mice can generate second messengers after BCR activation. Additional studies aimed at comparing *syk*^{-/-} and *BLNK*^{-/-} mice will be required to assess this possibility.

Finally, the developmental block at the pre-to pre-B cell transition observed in a BLNK-deficient patient is similar, though not identical, to the phenotype observed in *BLNK*^{-/-} mice (31). Although IgM^{hi} B cells accumulate in the periphery of *BLNK*^{-/-} mice, no peripheral B cells were detected in this adult patient. Similar discordance in phenotypes has been observed in immunodeficiencies involving Btk and $\lambda 5$ in which the human phenotype appears to be more severe than the murine phenotype (32). These differences may reflect a greater dependence on pre-BCR function in human B cell development, a species-specific difference in the regulation of signaling molecules that dictate activation thresholds, or both. Such species-specific differences have been observed in T cell development in which Syk is more highly expressed in developing human CD4⁺ T cells than in murine CD4⁺ T cells and may provide a mechanism to explain the phenotypic differences observed between ZAP-70-deficient mice and humans (33). Additional investigation is required to determine whether species-specif-

Fig. 4. Decreased maturation of B cell development and function in *BLNK*^{-/-} mice. (A) Splenocytes isolated from 3- to 5-week-old *BLNK*^{+/+} or *BLNK*^{-/-} mice were analyzed by FACS staining for IgM and IgD (27). (B) Splenocytes from older *BLNK*^{+/+} or *BLNK*^{-/-} mice (8 to 13 weeks old) were stained with the antibodies indicated in each figure and analyzed by FACS analysis (27). The percentages of gated cells are indicated. *BLNK*^{-/-} spleens were reduced in cell number by ~70% as compared to *BLNK*^{+/+} spleens (14, 18) (see text for absolute numbers of cells recovered). (C) Serum immunoglobulin levels of 8- to 13-week-old wild-type, germ line *BLNK*^{-/-} and R2:*BLNK*^{-/-} mice were determined by ELISA (23). Each diamond represents the value derived from an individual mouse. The black bar denotes the mean of each group. (D) B220⁺ B cells from older *BLNK*^{+/+} (solid line) and *BLNK*^{-/-} (dotted line) mice were analyzed by FACS analysis for increases in [Ca²⁺]_i after BCR cross-linking with antibody to IgM F(ab')₂ fragments (20 and 40 μ g/ml) (26). Antibody to IgM F(ab')₂ was added at time 0 at the indicated concentrations. Ionomycin was added at 5 min to ensure proper loading of cells with Fluo-4. Diamond, *BLNK*^{+/+} splenocytes; square, *BLNK*^{-/-} splenocytes. (E) B220⁺ B cells from older *BLNK*^{+/+} (top) and *BLNK*^{-/-} (bottom) mice were analyzed by FACS analysis for up-regulation of CD69 and CD86 expression after BCR cross-linking (27). The dotted line represents cells treated with media. The solid line represents cells treated with antibody to IgM F(ab')₂ (10 μ g/ml).



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References and Notes

1. R. Benschop and J. Cambler, *Curr. Opin. Immunol.* **11**, 143 (1999); K. Campbell, *Curr. Opin. Immunol.* **11**, 256 (1999); T. Kurosaki, *Annu. Rev. Immunol.* **17**, 555 (1999).
2. J. Healy and C. Goodnow, *Annu. Rev. Immunol.* **16**, 645 (1998).
3. C. Fu, C. Turck, T. Kurosaki, A. Chan, *Immunity* **9**, 93 (1998).
4. L. Gangi-Peterson et al., *Mol. Immunol.* **35**, 55 (1998); S. Hashimoto et al., *Blood* **94**, 2357 (1999).
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8. R. Hardy, C. Carmarck, S. Shinton, J. Kemp, K. Hayakawa, *J. Exp. Med.* **173**, 1213 (1991).
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22. F. Loder et al., *J. Exp. Med.* **190**, 75 (1999).
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25. J. G. Cyster et al., *Nature* **381**, 325 (1996).
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29. A. B. Kantor, A. M. Stall, S. Adams, L. A. Herzenberg, L. A. Herzenberg, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 3320 (1992).
30. H. von Boehmer et al., *Curr. Opin. Immunol.* **11**, 135 (1999).
31. Y. Minegishi et al., *Science* **286**, 1954 (1999).
32. S. Tsukada et al., *Cell* **72**, 279 (1993); D. Vetrie et al., *Nature* **361**, 226 (1993); W. N. Khan et al., *Immunity* **3**, 283 (1995); J. D. Kerner et al., *Immunity* **3**, 301 (1995); R. W. Hendriks et al., *EMBO J.* **15**, 4862 (1996); Y. Minegishi et al., *J. Exp. Med.* **187**, 71 (1998).
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An Essential Role for BLNK in Human B Cell Development

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Cross-linking of the B cell antigen receptor (BCR) results in rapid phosphorylation of the adapter protein BLNK [also called SLP-65 (Src homology 2 domain-containing leukocyte protein of 65 kD) and BASH (B cell adapter containing Src homology 2 domain)], a hematopoietic-specific cytoplasmic protein with ho-

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References and Notes

1. R. Benschoep and J. Cambier, *Curr. Opin. Immunol.* **11**, 143 (1999); K. Campbell, *Curr. Opin. Immunol.* **11**, 256 (1999); T. Kurosaki, *Annu. Rev. Immunol.* **17**, 555 (1999).
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9. A. M. Cheng et al., *Nature* **378**, 303 (1995); M. Turner et al., *Nature* **378**, 298 (1995); M. Turner et al., *J. Exp. Med.* **186**, 2013 (1997).
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BLNK-deficient DT40 chicken B cell line indicate that this adapter is required for the release of intracellular calcium and the activation of the extracellular signal-regulated protein kinase, c-Jun NH₂-terminal kinase, and p38 pathways in response to anti-immunoglobulin M (IgM) stimulation (3). If BLNK plays a nonredundant role in mammalian B cell development, mutations in BLNK might result in immunodeficiency.

About 85% of patients with early onset hypogammaglobulinemia and absent B cells are males with X-linked agammaglobulinemia (XLA) (4). These patients have mutations in the cytoplasmic tyrosine kinase Btk (5). Some of the remaining patients have defects in components of the pre-B cell receptor (pre-BCR) or BCR (6, 7); however, the nature of the defect in many patients remains unknown. To determine if mutations in BLNK could give rise to human immunodeficiency, we isolated and characterized a bacterial artificial chromosome clone containing the human genomic BLNK sequence. Fluorescence in situ hybridization demonstrated that BLNK is located on chromosome 10q23.22. The gene consists of 17 exons spread over ~65 kb of DNA. Primers were designed to amplify individual exons by polymerase chain reaction (PCR) for analysis by single-strand conformation polymorphism (SSCP) (8). Genomic DNA samples were analyzed from 25 patients with a Btk-deficient phenotype, in whom we had not identified mutations in Btk, μ heavy chain, Ig α (mb-1), Ig β (B29), or the surrogate light chain. DNA from one patient, a 20-year-old male with early onset hypogammaglobulinemia and absent B cells, demonstrated a homozygous alteration in the first exon of BLNK and its flanking intronic sequence (Fig. 1). This portion of the gene was cloned and sequenced, and two noncontiguous base-pair substitutions were identified (9). The first alteration, a C to A substitution, occurred at the third base-pair position in codon 10, which encodes a proline. This base-pair substitution does not change the amino acid sequence of BLNK. The second alteration, an A to T substitution, was found at the +3 position of the splice donor site for intron 1, 20 base pairs downstream from the alteration in codon 10. SSCP analysis of DNA from 100 unrelated individuals did not reveal any fragments with a migration pattern identical to that seen in the patient (10).

The A to T substitution at the +3 position of the splice donor site occurs at a highly conserved site in the splice consensus sequence; alterations at this site would be expected to result in faulty processing of the BLNK message (11). To evaluate this possibility, we derived cDNA from the patient's bone marrow and used reverse transcriptase-PCR (RT-PCR) to examine the abundance of BLNK transcripts (12). The results were compared with those obtained from bone marrow of healthy subjects or patients with mutations in Btk or μ heavy chain (Fig. 2). No BLNK transcripts could be amplified from the patient's bone marrow, although BLNK transcripts were easily identified in the bone marrow of the other patients with defects in early B cell development. Other genes expressed in pro-B cells, including Btk, terminal deoxynucleotidyl transferase (TdT), and λ 5, were expressed in approximately equal amounts in all of the patients. These results indicate that the base-pair substitutions in BLNK resulted in a marked reduction or absence in BLNK transcripts and therefore in BLNK protein.

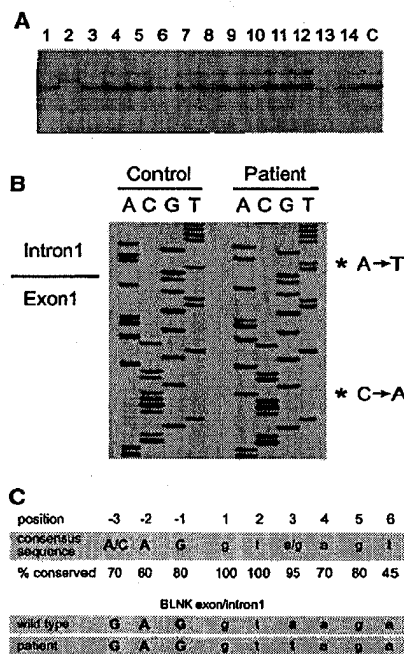


Fig. 1. Characterization of the BLNK mutation in an immunodeficient patient. (A) Genomic DNA samples from 14 patients with defects in B cell development and a control (lane C) were analyzed by SSCP for defects in the first exon of BLNK and its flanking sequences. DNA from the patient is shown in lane 2. (B) Sequence analysis at the exon-intron border demonstrated two base-pair substitutions, as indicated. (C) The consensus sequence for a mammalian 5' splice donor site is shown with the wild-type and mutant BLNK exon 1/intron 1 sequence. The coding sequence is shown in capital letters; the intronic sequence is in lowercase letters.

The patient with BLNK deficiency demonstrated normal growth and development. At 8 months of age, he had the onset of recurrent otitis. After two episodes of pneumonia, he was evaluated for immunodeficiency at 16 months of age. At that time, he had no detectable serum IgG, IgM, or IgA, and he had <1% B cells in the peripheral circulation. He was started on gammaglobulin replacement, and between 2 and 20 years of age, he did well except for chronic otitis and sinusitis, hepatitis C acquired from intravenous gammaglobulin, and an episode of protein-losing enteropathy in adolescence. Immunologic studies performed when the BLNK-deficient patient was 20 years of age demonstrated serum concentrations of IgM and IgA of <7 mg/dl, normal numbers and percentages of CD4 and CD8⁺ T cells and natural killer cells, and normal numbers of platelets and myeloid cells. The patient's mother and father, who were heterozygous for both base-pair substitutions in BLNK, were healthy and had normal concentrations of serum immunoglobulins and normal numbers of B cells (13). An older brother developed recurrent otitis at 6 months of age and died at 16 months of age of pseudomonas sepsis and neutropenia.

Immunofluorescence analysis of peripheral blood lymphocytes from the patient with BLNK deficiency and an age-matched patient with an amino acid substitution in the pleckstrin homology domain of Btk demonstrated that both patients had <0.01% CD19⁺ cells in the blood (14). To determine the point in B cell differentiation at which the block in develop-

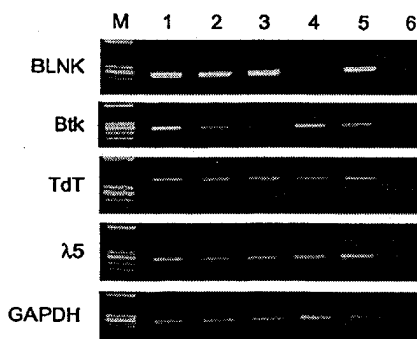


Fig. 2. RT-PCR analysis of B cell-specific transcripts in patients with defects in B cell development. RNA from the following sources was reverse transcribed: the bone marrow of a normal control (lane 1), a patient with an amino acid substitution in codon 113 of Btk (lane 2), a patient with a 4-bp deletion in the coding sequence of Btk (lane 3), the patient with mutations in BLNK (lane 4), and a patient with an amino acid substitution at an invariant cysteine in CH4 of μ heavy chain (lane 5). The cDNA was used as a template for RT-PCR with primers specific for the coding regions of BLNK, Btk, TdT, λ 5, and the control transcript, GAPDH. Molecular weight markers are shown on the left (lane M), and a cDNA negative control is shown on the right (lane 6).

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ment occurred, we examined bone marrow from both patients using markers that distinguish pro-B cells from pre-B cells and mature B cells. The percentage of CD19⁺ B lineage cells was less in the patients in comparison to that of the control (0.3% in the BLNK-deficient patient and 1.0% in the Btk-deficient patient versus 15.7% in the control). There were no membrane immunoglobulin-positive (mIg⁺) mature B cells in either patient (Fig. 3). In both patients, the block in B cell differentiation occurred at the pro-B cell to pre-B cell transition; >80% of the CD19⁺ cells from these patients coexpressed the pro-B cell marker, CD34. In contrast, only 22.0% of the CD19⁺ cells from the control were positive for CD34; the remaining

cells from the control were either pre-B cells (CD34⁻, CD19⁺, and mIg⁻) or B cells (CD34⁻, CD19⁺, and mIg⁺).

To document that BLNK is expressed in pro-B cells, we indirectly stained permeabilized bone marrow cells from the Btk- and BLNK-deficient patients with a monoclonal antibody to BLNK (14). All of the CD19⁺ pro-B cells from the Btk-deficient patient were positive for BLNK (Fig. 4). By contrast, there was little or no staining for BLNK in the bone marrow of the patient with mutations in BLNK. Because BLNK is expressed in pro-B cells, the possibility that BLNK is required before the expression of the pre-BCR was examined. In previous studies, we have shown that patients with de-

fects in the constant region of the μ heavy chain or the Ig α signal transduction component of the BCR have small amounts of transcripts for rearranged μ heavy chain genes in the bone marrow as detected by RT-PCR (7). Rearrangement of the μ heavy chain occurs immediately before the pro-B cell to pre-B cell transition. A primer that hybridizes to a conserved sequence within framework region 3 of variable-region genes and a primer within the CH1 domain of μ heavy chain were used to examine cDNA from a control and patients with defects in B cell development (7). A small number of rearranged μ heavy chain transcripts could be detected in the bone marrow of the patient with mutations in BLNK as well as in patients that were Btk and μ heavy chain deficient. Thus, BLNK does not play a role in B cell development before the expression of the pre-BCR. This corresponds with earlier studies showing that phosphorylation of BLNK is dependent on cell surface expression of a BCR (15).

Cell surface expression of the pre-BCR results in a strong survival signal associated with the cessation of μ heavy chain gene rearrangements, changes in cell surface phenotype, and marked expansion of the pre-B cell population (16). The absence of pre-B cells or B cells in the patient with mutations in BLNK demonstrates that BLNK plays a critical role in orchestrating these signals. Like defects in Btk and $\lambda 5$ (5, 6, 17), mutations in BLNK appear to have more severe consequences in the human as compared to the mouse (18). This suggests that the requirements for signaling through the pre-BCR and BCR may be more stringent in the human than in the mouse. There may be a reciprocal reliance on signaling through other pathways in murine B cell development. For example, the consequences of defective signaling through interleukin-7 are more severe in the mouse as compared to the human (19).

In T cells, the functions performed by BLNK appear to be split between LAT (linker for activation of T cells), which binds to phosphatidylinositol 3-kinase, Grb-2, and PLC γ (20), and SLP-76, which binds GrpL, Nck, Vav, and Fyb (FYN binding protein) (21). Mice lacking LAT (22) or SLP-76 (23, 24) have a block in T cell development at the pro-T to pre-T cell stage of development. In the newborn period, SLP-76-deficient mice also develop a hemorrhagic diathesis, which is related to the requirements for SLP-76 in collagen-mediated platelet activation (24, 25). These studies, when coupled with our findings showing that BLNK is required for normal B cell development in the human and the mouse, indicate that adapter proteins play a critical role in highly specific signaling pathways, and they suggest that defects in adapter proteins like LAT or SLP-76 may result in human immunodeficiency.

Fig. 3. Immunofluorescence analysis of B lineage cells. Bone marrow mononuclear cells from a normal individual (left column), from a patient with Btk-deficient XLA (middle column), and from the BLNK-deficient patient (right column) were labeled with antibody to CD19 PE, antibody to CD34 PerCP, and antibody to Ig κ and λ light chains FITC. Flow cytometric dot plots in the top row illustrate CD19 staining versus side scatter (SSC); both patients had reduced proportions of CD19⁺ lymphoid cells were then analyzed for expression of mIg light chains (middle row) and CD34 (bottom row). Percentages of mIg⁺ and CD34⁺ among CD19⁺ cells are indicated.

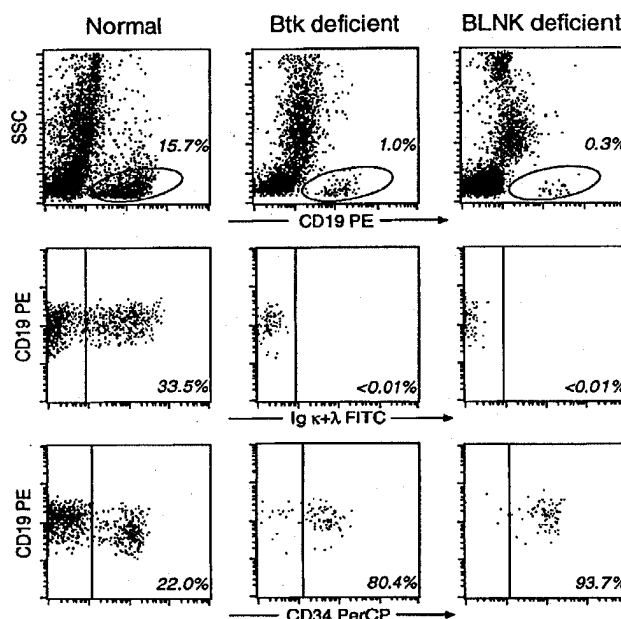
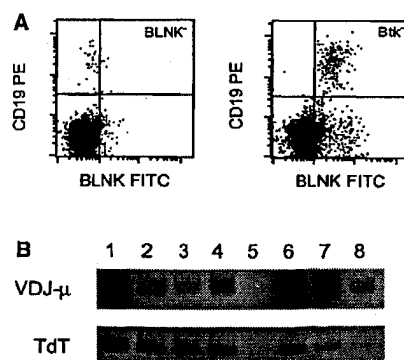


Fig. 4. (A) BLNK protein expression in pro-B cells. Bone marrow mononuclear cells from age-matched patients with BLNK (left) or Btk (right) deficiency were labeled with antibody to CD19 (IgM), permeabilized, and labeled with monoclonal antibody to BLNK (IgG2a). Goat antibody to mouse IgM PE and IgG FITC was then added. Dot plots illustrate immunofluorescence staining of lymphoid cells. Quadrants were set at the upper limits of the isotype-matched nonreactive antibody fluorescence. The BLNK⁺ CD19⁻ cells seen in the Btk-deficient patient were CD34⁻ and were similar to monocytes in forward and side light scatter. (B) Semiquantitative RT-PCR analysis to evaluate the amount of VDJ-rearranged μ heavy-chain transcripts. Bone marrow cDNA from an age-matched control (lane 1), a patient with XLA (lane 2), the patient with BLNK deficiency (lane 3), a patient with μ heavy chain deficiency (lane 4), and a cDNA negative control (lane 5) and three 10-fold dilutions of control cDNA (1 \times , 0.1 \times , and 0.01 \times) (lanes 6 through 8, respectively) were amplified with primers specific for VDJ-rearranged μ heavy chain and TdT, which was used as a control to demonstrate equal concentrations of pro-B cell transcripts.



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References and Notes

- C. Fu, C. W. Turck, T. Kurosaki, A. C. Chan, *Immunity* 9, 93 (1998).
- J. Wienands et al., *J. Exp. Med.* 188, 791 (1998); R. Goltsuka et al., *J. Immunol.* 161, 5804 (1998).
- M. Ishiai et al., *Immunity* 10, 117 (1999).
- M. E. Conley, D. Mathias, J. Treadaway, Y. Minegishi, J. Rohrer, *Am. J. Hum. Genet.* 62, 1034 (1998).
- S. Tsukada et al., *Cell* 72, 279 (1993); D. Vetrie et al., *Nature* 361, 226 (1993).
- L. Yel et al., *N. Engl. J. Med.* 335, 1486 (1996); Y. Minegishi et al., *J. Exp. Med.* 187, 71 (1998).
- Y. Minegishi et al., *J. Clin. Invest.* 104, 1115 (1999).
- The sequence flanking each exon of BLNK has been deposited in GenBank (accession numbers AF180740 through AF180756). The primers used to amplify each exon are available upon request. The conditions used in SSCP are as described in work by M. E. Conley, M. E. Fitch-Hilgenberg, J. L. Cleveland, O. Parolini, and J. Rohrer [*Hum. Mol. Genet.* 3, 1751 (1994)].
- The following primers were used to amplify BLNK exon 1 and the associated flanking sequence for both the SSCP analysis and cloning of this region of the gene: 5'-GAAGCTGCTGACGTGACCA-3' (5' untranslated region of exon 1) and 5'-CCCTAAAGCTCAGTCCAC-3' (intron 1). The products of two independent PCR reactions were cloned and sequenced.
- DNA samples from five individuals demonstrated altered migration in comparison to the wild-type pattern. Sequencing showed a T to A substitution at the +113 position in intron 1 in three people, a G to A substitution at the +121 position in intron 1 in one individual, and an A to G substitution 5 base pairs upstream of the start codon.
- D. N. Cooper and M. Krawczak, *Human Gene Mutation* (BIOS, Oxford, 1994), pp. 239-260.
- The primers used to amplify BLNK cDNA were from the 5' untranslated region [shown in (9)] and 5'-CTCGCTGCTCAAGTCATCGGA-3' from exon 4. The Btk-specific primers were from exons 6 and 8 of that gene. The primers used to amplify Tdt, λ 5, VDJ- μ , and glyceraldehyde phosphate dehydrogenase (GAPDH) were as reported in (7).
- The father had 6% and the mother had 8% CD19⁺ B cells (normal is 5 to 22%). Serum IgG in the father was 1110 mg/dl, IgA was 337 mg/dl, and IgM was 93 mg/dl. The mother's IgG was 1060 mg/dl, IgA was 115 mg/dl, and IgM was 127 mg/dl.
- Bone marrow mononuclear cells were stained with antibody to CD19 conjugated to phycoerythrin (PE) (Dako, Carpinteria, CA), antibody to CD34 conjugated to peridinin chlorophyll protein (PerCP) (Becton-Dickinson, San Jose, CA), and polyclonal antibodies against human light chains conjugated to fluorescein isothiocyanate (FITC) (Southern Biotechnology Associates, Birmingham, AL). Antibodies to nuclear Tdt (Supertechs, Bethesda, MD) and cytoplasmic μ heavy chains (Southern Biotechnology Associates) conjugated to FITC and PE, respectively, were applied after cell permeabilization with OrthoPermeafix (Ortho Diagnostics, Raritan, NJ). Monoclonal antibody to BLNK (of IgG2a isotype) was generated against a glutathione S-transferase fusion protein encoding amino acids 4 through 205, as described in (1). This antibody was used in combination with an antibody to CD19 of IgM class (BLY3) (Research Diagnostics, Flanders, NJ), with fluorochrome-conjugated secondary antibodies specific for murine IgG and IgM. Antibody to BLNK was applied after cell permeabilization with OrthoPermeafix. Isotype-matched nonreactive antibodies were from Becton-Dickinson. Immunofluorescence staining was analyzed with a FACScan flow cytometer equipped with CellQuest software (Becton-Dickinson).
- Y. Zhang, J. Wienands, C. Zurn, M. Reth, *EMBO J.* 17, 7304 (1998).
- K. Rajewsky, *Nature* 381, 751 (1996).
- W. Khan et al., *Immunity* 3, 283 (1995); J. D. Kenner et al., *Immunity* 3, 301 (1995); D. Kitamura et al., *Cell* 69, 823 (1992).
- R. Pappu et al., *Science* 286, 1949 (1999).
- J. J. Peschon et al., *J. Exp. Med.* 180, 1955 (1994); A. Puel, S. F. Ziegler, R. H. Buckley, W. J. Leonard, *Nature Genet.* 20, 394 (1998).
- W. Zhang, J. Sloan-Lancaster, J. Kitchen, R. P. Tribble, L. E. Samelson, *Cell* 92, 83 (1998).
- J. K. Jackman et al., *J. Biol. Chem.* 270, 7029 (1995); D. G. Motto, S. E. Ross, J. Wu, L. R. Hendricks-Taylor, G. A. Koretzky, *J. Exp. Med.* 183, 1937 (1996); L. Tuosto, F. Michel, O. Acuto, *J. Exp. Med.* 184, 1161 (1996); A. J. da Silva et al., *Proc. Natl. Acad. Sci. U.S.A.* 94, 7493 (1997); A. Marie-Cardine et al., *J. Biol. Chem.* 273, 25789 (1998); S. K. Liu, N. Fang, G. A. Koretzky, C. J. McGlade, *Curr. Biol.* 9, 67 (1999); C. L. Law et al., *J. Exp. Med.* 189, 1243 (1999); L. Wunderlich, A. Farago, J. Downward, L. Buday, *Eur. J. Immunol.* 29, 1068 (1999).
- W. Zhang et al., *Immunity* 10, 323 (1999).
- J. L. Clements et al., *Science* 281, 416 (1998).
- V. Pivniouk et al., *Cell* 94, 229 (1998).
- J. L. Clements et al., *J. Clin. Invest.* 103, 19 (1999).
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Perforin Gene Defects in Familial Hemophagocytic Lymphohistiocytosis

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Familial hemophagocytic lymphohistiocytosis (FHL) is a rare, rapidly fatal, autosomal recessive immune disorder characterized by uncontrolled activation of T cells and macrophages and overproduction of inflammatory cytokines. Linkage analyses indicate that FHL is genetically heterogeneous and linked to 9q21.3-22, 10q21-22, or another as yet undefined locus. Sequencing of the coding regions of the perforin gene of eight unrelated 10q21-22-linked FHL patients revealed homozygous nonsense mutations in four patients and missense mutations in the other four patients. Cultured lymphocytes from patients had defective cytotoxic activity, and immunostaining revealed little or no perforin in the granules. Thus, defects in perforin are responsible for 10q21-22-linked FHL. Perforin-based effector systems are, therefore, involved not only in the lysis of abnormal cells but also in the down-regulation of cellular immune activation.

FHL is a hemophagocytic lymphohistiocytic disorder in which previously healthy young children present with fever, splenomegaly, hepatomegaly, pancytopenia, coagulation abnormalities, neurological abnormalities, and high serum concentrations of interferon- γ (IFN- γ) and tumor necrosis factor- α (TNF- α). Accumulation of activated macrophages and lymphocytes, mainly CD8⁺ human lymphocyte antigen DR⁺ Fas⁺ T cells, as well as hemophagocytosis in the bone marrow, spleen, liver, lymph nodes,

and central nervous system, dominate the pathology (1-3). Defective T and natural killer (NK) cell cytotoxicity is consistently reported (4, 5). We hypothesized that the primary inherited defect in FHL could be a failure of cytolytic lymphocyte function and that this, together with childhood infections (6, 7), induces the fatal immune deregulation of FHL.

The gene encoding perforin, an important mediator of lymphocyte cytotoxicity, has been mapped to 10q22 (8), near one of the previously identified FHL-linked loci (9, 10). Thus, perforin deficiency may play a role in the pathogenesis of FHL. Unlike patients with FHL, perforin knockout mice are generally healthy when maintained in a pathogen-free, controlled environment. However, when infected with lymphocytic choriomeningitis virus (LCMV), similar CD8⁺ T cell-, IFN- γ -, TNF- α -dependent immunopathology and mortality are seen (11, 12).

We first confirmed the presence of perforin (PRF1) in the candidate region by polymerase chain reaction (PCR) screening a partial yeast artificial chromosome (YAC) contig covering the FHL region on chromosome 10 using prim-

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